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Optimization of Operating Conditions for the Production of L-asparaginase by *Enterobacter aerogenes* MTCC 2823 using Central Composite Design

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ABSTRACT:
Statistical experimental designs such as response surface methods can develop high efficiency and economic bioprocess. Hence in the present work effect of operating conditions such as agitation rate, pH and temperature on L-asparaginase production by *Enterobacter aerogenes* MTCC 2823 in shake culture fermentation was studied and optimized using central composite design. The experimental value of L-asparaginase activity was subjected to multiple linear regression analysis using MINITAB 15 software. A second order polynomial model was developed using coded units of the independent variables and the optimum value of agitation rate, pH and temperature was estimated from second order polynomial model using MINITAB 15 software. The optimum value of agitation rate, temperature and pH was 186 rpm, 35°C and 6.7 respectively for maximum L-asparaginase activity of 20.17 IU/mL.

Keywords: Fermentation, polynomial model, multiple linear regression, response surface method.

INTRODUCTION

The manufacture of enzyme for use as drug is important facet of today's pharmaceutical industry. L-asparaginase (L-asparagine amidohydrolase; EC.3.5.1.1) is used as chemotherapeutic agent for the treatment of acute *lumphocytic leukeamia* and less frequently for acute *myeloblastic leukeamia*, chronic *lumphocytic leukeamia*, Hodgkin's disease, *melonosarcoma* and non-Hodgkin's *lymphoma* [1].

Tsuji first reported the Deamidation of L-asparagine by extracts of E. coli in 1957. Broome in 1961 discovered that the regression of *lymphosarcoma* transplants in mice treated with guinea-pig serum was due to the nutritional dependence of the malignant cells on exogenous L-asparagine.

Commercial production of L-asparaginase appeared desirable only after Mashburn and Wriston in 1973 showed that L-asparaginase from *E. coli* inhibits tumors in mice [2]. Various bacterial cultures, such as, *Erwinia carotovora, Thermus thermophilus [3], Thermus aquaticus, Vibrio succinogenes, Citrobacter freundi, Streptomyces griseus, Escherichia coli, Erwinia aroideae, Proteus vulgeris, Enterobacter aerogenes, Zymomonas mobilis [4], Bacillus licheniformis, Pseudomonas aeruginosa [5], Enterobacter aerogenes* [6, 7] have been found to produce L-asparaginase.
The optimization of nutritional requirements and operating conditions is an important step in any bioprocess development. Statistical experimental have been used in several steps of optimization strategy and it is better acknowledged than traditional one variable at a time method [8, 9].

The Response Surface Methodology (RSM) is an empirical modeling and optimization system that assesses the relationship between a group of variables, which can be controlled experimentally. RSM is an efficient statistical technique for the optimization of multiple variables in order to predict the best performance conditions with a minimum number of experiments [10-12].

These designs are used to find improved or optimal process settings, troubleshoot process problems and weak points and make a product or process more robust against external and non-controllable influences.

In this work a full factorial Central Composite Design (CCD) was applied to determine the optimum level of process conditions such as agitation rate (rpm), pH and temperature for the production of L-asparaginase by

IJBST

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Enterobacter aerogenes MTCC 2823 in shake culture fermentation.

MATERIALS AND METHODS

Microorganism

The bacteria Enterobacter aerogenes MTCC 2823, obtained from Institute of Microbial Technology, Chandigarh, India. It was grown on yeast extract-peptone nutrient agar slants for 24 hr at 30°C, and maintained at 4°C as stock culture and subcultured for every month.

Preparation of Inoculum Culture

Liquid media with following composition in % were prepared. Peptone 1, Yeast extract 0.5, L-asparagine 0.5, Potassium Chloride 0.05; $MgSO_4.7H_2O$, 0.05; $FeSO_4.7H_2O$, 0.001; K_2HPO_4 0.1; and 1 mL of 20% Glucose solution per 100mL of liquid media was prepared and it was inoculated with Stock culture of *Enterobacter aerogenes* MTCC 2823 and grown at pH 7 and temperature of 30°C for 24 h.

Production of L-asparaginase

Production media was prepared with following components in % (gm/100mL), Sodium nitrate 0.2, Sodium citrate 1, L-asparagine 0.5, glucose 0.5, Yeast extract 0.5, L-asparagine 0.5, Potassium Chloride 0.05; $MgSO_4.7H_2O$, 0.05; $FeSO_4.7H_2O$, 0.001; K_2HPO_4 0.1; 50 mL of media was inoculated with 5 % liquid inoculum culture of *Enterobacter aerogenes* MTCC 2823.

Cultures were grown at agitation rate, pH and temperature as designed by central composite design shown in table 1. A culture sample of 2mL was collected at maximum L-asparaginase production time of 6 h [6] and centrifuged at 10000 rpm at 5°C and the cell mass was used to analyze intracellular L-asparaginase activity.

Assay of L-asparaginase Activity

The cell mass were shaken vigorously with 20 ml phosphate buffer (pH 7.0) containing triton X-100 (0.01%) for 5 min and centrifuged 10000 rpm. The cells were suspended in 1.5 ml sodium borate buffer pH 8.65, and L-asparaginase activity was assayed by Nesslarization, most commonly used method for estimating L-asparaginase activity [6].

Optimization by Central Composite Design

The three variables such as agitation rate (rpm), pH and temperature were identified as important operating conditions in the production of *Enterobacter aerogenes* from literature study. Variables were prescribed into three levels, -1, 0, + 1 for low, middle and high and central composite experimental design was developed as shown Table 1 using Minitab15 software.

The coded and actual (Xi) level of variables is shown in Table 2. All the experiments were carried out in duplicate and average of the results were analyzed. Based on the regression analysis a second order polynomial model describes the relationship between the independent variables and L-asparaginase activity as given in equation 1 was developed. The closer the value of composite desirability to 1, the predicted values and model was well fitted with the experimental results [2, 9].

The optimum levels of the variables were obtained by solving the regression equation and also by analyzing the response surface plots.

$$Y= \beta_0+\beta_1X_1+\beta_2X_2+\beta_3X_3+\beta_{11}X_1^2+ \beta_{22}X_2^2+\beta_{33}X_3^2+\beta_{12}X_1X_2+ \beta_{13}X_1X_3+\beta_{23}X_2X_3 \quad \ldots. (1)$$

RESULTS AND DISCUSSION

All the experiments were carried out in duplicate and average L-asparaginase activity given in table 1 was subjected to multiple linear regression analysis using MINITAB 15 software. The effect of agitation rate, temperature and pH on L-asparaginase activity was described in the form second order polynomial model in coded units (equation 2).

Table 1. Experimental range and levels of operating conditions

Independent variable	Level and Range				
	-1.681	-1	0	1	1.681
agitation rate (X_1) rpm	99.5	120	150	180	200.5
Temperature (X_2) ºC	26.6	30	35	40	43.4
pH (X_3)	5.3	6	7	8	8.7

IJBST

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Table 2. Central composite Experimental design in coded units and L-asparaginase activity

Std. Exp. No.	Agitation rate (X_1)	Temperature (X_2)	pH (X_3)	L-asparaginase activity (IU/mL)	
				Experimental	Predicted
1	-1	-1	-1	11.195	10.845
2	1	-1	-1	21.086	16.088
3	-1	1	-1	12.717	11.138
4	1	1	-1	14.508	16.841
5	-1	-1	1	20.815	17.865
6	1	-1	1	13.803	14.765
7	-1	1	1	10.760	15.141
8	1	1	1	12.770	12.502
9	-1.681	0	0	17.226	17.225
10	1.681	0	0	18.540	19.414
11	0	-1.681	0	10.487	14.551
12	0	1.681	0	16.086	12.894
13	0	0	-1.681	8.532	10.966
14	0	0	1.681	14.781	13.220
15	0	0	0	19.943	19.883
16	0	0	0	19.836	19.883
17	0	0	0	19.943	19.883
18	0	0	0	19.998	19.883
19	0	0	0	19.672	19.883
20	0	0	0	20.054	19.883

Student's t-test was performed to determine the significance of regression coefficients. The regression coefficient, t and p values for linear, quadratic and combined effects were given in the Table 3, with a 95% significance level.

It was observed that the coefficients for over all effect of variables and quadratic effect of pH were highly significant ($p = 0.000$ & $p = 0.007$) than linear effect of agitation rate and temperature and combined effects between parameters. Higher value of coefficient of correlation justified an excellent correlation between aeration rate, temperature and pH and the model fitted well with L-asparaginase activity and optimal values.

$$Y_{activity} = 19.881+0.651X_1-0.493X_2+0.670X_3- 0.553X_1^2-2.177X_2^2-2.754X_3^2+ 0.115X_1X_2-.085X_1X_3-0.754X_2X_3 \quad ... (2)$$

Response surface plots described by the regression model for CCD were developed using MINITAB 15 software. Figure 1 shows the effect of temperature and pH on L-asparaginase activity, while other variable (agitation rate) was fixed at lower level.

It was observed that the L-asparaginase activity was less at lower and higher level of temperature and pH, L-asparaginase activity was increased at middle level of temperature and pH with maximum L-asparaginase activity to 20 IU/mL. Figure 2 shows the effect of agitation rate (rpm) and pH on L-asparaginase activity, while other variable (temperature) was fixed at lower level. It was observed that the L-asparaginase activity was less at lower and higher level of pH and L-asparaginase activity was increased at higher level of agitation rate and pH with maximum L-asparaginase activity to 20 IU/mL.

Figure 3 shows the effect of agitation rate (rpm) and temperature on L-asparaginase activity, while other variable (pH) was fixed at lower level. It was observed that the L-asparaginase activity was less at lower and higher level of agitation rate (rpm) and temperature; L-asparaginase activity was increased at middle level of agitation rate (rpm) and temperature with maximum L-asparaginase activity to 20 IU/mL.

Figure 4 shows that lower level of agitation rate increases the L-asparaginase activity and no effect at higher level, and temperature and pH increases the L-asparaginase activity at their optimum level.

The optimal value variables estimated in uncoded units as aeration rate186 rpm, temperature 35°C, and pH 6.7 with predicted L-asparaginase activity of 20.17 IU/mL. The composite desirability of 92.73 % at optimal condition reveals the high accuracy of the regression model.

IJBST

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Table 3. Estimated regression coefficients of second order polynomial model for the effect of operating conditions on the production of L-asparaginase by *Enterobacter aerogenes* MTCC 2823

Factor	Estimated coefficient	Standard deviation	t- value	p-value
	19.881	1.275	15.584	0.000
X1	0.651	0.846	0.769	0.460
X2	-0.493	0.846	-0.582	0.573
X3	0.670	0.846	0.792	0.447
X12	-0.553	0.824	-0.671	0.518
X22	-2.177	0.824	-2.643	0.025
X32	2.754	0.824	-3.342	0.007
X1X2	0.115	1.106	0.104	0.919
X1X3	-2.085	1.106	-1.886	0.089
X2X3	-0.754	1.106	-0.682	0.511

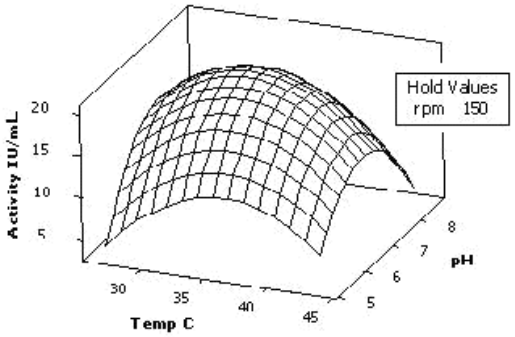

Fig.1. Response surface plot shows the interaction effect of temperature and pH on L-asparaginase activity.

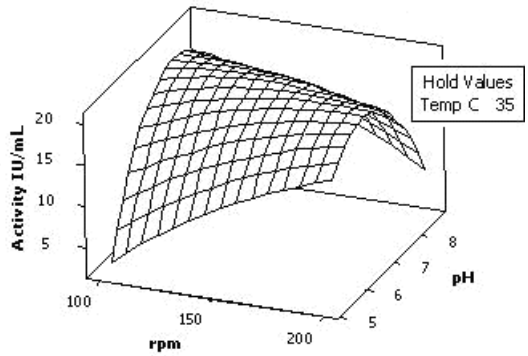

Fig.2. Response surface plot shows the interaction effect of agitation rate (rpm) and pH on L-asparaginase activity.

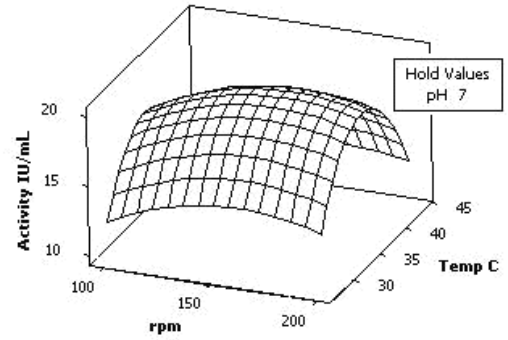

Fig.3. Response surface plot shows the interaction effect of agitation rate and temperature on L-asparaginase activity.

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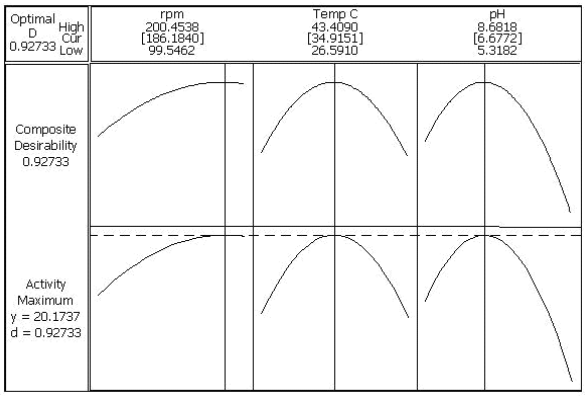

Fig.4. Composite desirability and optimization plot for maximum L-asparaginase activity

CONCLUSION

It is evident that the use of response surface methodology has helped to locate the optimum levels of operating conditions for maximal L-asparaginase production. This study shows that increase in agitation rate, temperature and pH promotes an increase in L-asparaginase activity with the optimal values of aeration rate, temperature and pH was estimated in actual units were aeration rate186 rpm, temperature 35°C, and pH 6.7 at predicted L-asparaginase activity of 20.17 IU/mL. The composite desirability of 92.73 % reveals the validity of the predicted values with experimental results.

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